# Phenoloxidase and peroxidase activities in *Sphagnum*-dominated peatland in a warming climate

Vincent E.J. Jassey, Geneviève Chiapusio, Daniel Gilbert, Marie-Laure Toussaint and Philippe Binet

Laboratoire Chrono-Environnement, UMR CNRS 6249, UFR Sciences, techniques et gestion de l'industrie, Université de Franche-Comté, F-25211 Montbéliard cedex, France.

Correspondance to Philippe Binet

Laboratoire Chrono-Environnement, UMR CNRS 6249, UFR Sciences, techniques et gestion de l'industrie, Université de Franche-Comté, 4 place Tharradin, Montbéliard 25211 cedex, France

Tel: +33 3 81 99 46 89; fax: +33 3 81 99 46 61

E-mail address: philippe.binet@univ-fcomte.fr

## Abstract

Peatlands still suffer from the scarcity of available data about the characterization and the response to climate forcing of the main oxidative enzymes that occur over the seasons. In the present study, phenoloxidase and peroxidase activities were examined in *Sphagnum* lawns along a narrow fen-bog gradient under experimental elevated temperatures. We showed that peroxidase activities from *Sphagnum* mosses were 1000-fold higher than those of phenoloxidases irrespective of seasons and sampling areas. Peroxidase activities increased (+ 30%) with the rise of air temperatures (an average of 1°C), while warming did not alter phenoloxidase activities. These results suggest that the monitoring of peroxidase activities in peatlands may represent a suitable and forward indicator of the impact of climate warming on carbon cycle in peatlands.

**Keywords:** 2,7-diaminofluorene; peroxidases; phenoloxidases; climate warming; peatland; open top chambers

Extracellular phenoloxidase and peroxidase measurements in ecosystems provide essential information on the stability of the carbon cycle (Sinsabaugh, 2010; Theuerl et al., 2010). By contributing to the oxidation and transformation of both complex and simple phenolic compounds, these enzymes induce partial or complete degradation of such recalcitrant compounds, and finally act on carbon cycling (Baldrian, 2006; Sinsabaugh, 2010; Theuerl et al., 2010). Considering the ongoing global warming, these enzymes gain scientific concern in terrestrial carbon reservoirs, such as peatlands (Fenner et al., 2005; Laiho, 2006; Jassey et al., 2011b). The accumulation of carbon in peat soils is thought to partly result from a suppression of the normal pathways of enzymatic decomposition in which oxidative enzymes, such as phenoloxidases, play a key role (Freeman et al., 2001, 2004).

Although phenoloxidases (PO) involved in the degradation of polyphenols are divided into PO $O_2$ (e.g. laccases, tyrosinases) and PO $H_2O_2$ (e.g. lignin and manganese peroxidases) dependent (Criquet et al., 2000a; Duran & Esposito, 2000; Sinsabaugh et al., 2003, Alarcón-Gutiérrez et al., 2008; Sinsabaugh, 2010), only phenoloxidase $O_2$ dependent have been predominantly investigated to date in peatlands. In forest litters, different spatiotemporal variations of PO $O_2$ and $H_2O_2$ dependent were recorded, emphasizing that all of these extracellular enzymes do not respond similarly to environmental changes (Criquet et al., 2000a; Duran and Esposito, 2000; Alarcón-Gutiérrez et al., 2008; Kaiser et al., 2010). Thus, peatlands suffer from the scarcity of available data about the characterization and the response to warming of these oxidative enzymes that occur over the seasons and ecological settings.

The purpose of the present paper was to determine the impact of an experimental climate warming on phenoloxidase activities $O_2$ and $H_2O_2$ dependent in peatlands over two seasons along a transitional fen-bog gradient. Because our assays did not discriminate individual enzymes, the generic terms phenoloxidase and peroxidase were chosen to describe the activity of enzymes that use $O_2$ and $H_2O_2$ as an acceptor, respectively.

57 During field campaigns of summer and autumn 2010, peroxidases and phenoloxidases
58 were investigated within a larger mire complex in fen and bog areas situated in the Jura
59 Mountains (France, 46°49'35''N, 6°10'20''E). Sampling areas were situated along a
60 transitional gradient between a poor fen and a raised bog with vegetation composition
61 dominated by *Sphagnum fallax* (Jassey et al., 2011b). Samples of *S. fallax* were collected and
62 cut into two levels: 0-3 cm (living segments = Top) and 3-10 cm (early declining segments =
63 Bottom) from the capitulum. In fen and bog areas, 6 *Sphagnum* plots were selected in
64 representative surfaces including 3 replicates as ambient treatment and 3 replicates as
65 warming treatment. The beginning of the warming treatment was on April 2008. Increasing of
66 air temperature was passively achieved in warming plots using open-top chambers (hereafter
67 referred as OTC) over the vegetation (Jassey et al., 2011b). Air temperatures (10 cm above
68 *Sphagnum* surface) were monitored continuously in each plot.

69 Because soil organic matter could affect enzyme activities, a specific method of
70 extraction was used (Criquet et al., 1999). 3 g FW of *S. fallax* in 50 mL 0.1 M $CaCl_2$ with
71 0.05% Tween 80 and 20 g PVPP were shaken for 1h. After centrifugation, the supernatant
72 was filtrated (0.2 µm) and concentrated in cellulose dialysis tubing (10 kDa molecular mass
73 cut-off) covered with polyethylene glycol. Then, concentrated extracts were resuspended in
74 phosphate buffer (pH 5.6) until 1/10 of the initial volume. Enzyme activities were measured
75 by spectrophotometry using a 96 wells microtiter plate. For phenoloxidase quantification,
76 each replicates wells contained 150 µL of enzyme-extract with, either 100 µL of L-DOPA (10
77 mM), or 2 µL of 2,7-diaminofluorene (DAF; 0.68 mM; $\varepsilon^M$ = 10 228 $M^{-1}.cm^{-1}$), or 2 µL of
78 syringaldazine (5 mM; $\varepsilon^M$ = 65 000 $M^{-1}.cm^{-1}$) or 5 µL of ABTS (0.1 mM; $\varepsilon^M$ = 36 000 $M^{-1}.cm^{-1}$)
79 ) in assay wells, and monitored at 460, 600, 525 and 420 nm, respectively (Criquet et al.,
80 2000a; Jassey et al., 2011b). Peroxidase activities were measured using 2 µL DAF (0.68 mM)
81 with 10 µL of $H_2O_2$ (0.3%), and manganese peroxidases (Mn-peroxidases) with 12 µL of
82 $MnSO_4$ (0.1 mM) (Criquet et al., 2001). Their oxidation rate was monitored at 600 nm.

Peroxidase activity was subtracted to the Mn-peroxidase assay to obtain the Mn-peroxidase activity. We also quantified fungal lignin-peroxidases using 12 µL veratrylic alcohol (0.4 mM; $\varepsilon^{M}$ = 9300 $M^{-1}.cm^{-1}$) with 10 µL of $H_2O_2$ (0.3%) and monitored at 310 nm in quartz cuvettes (Tien and Kirk, 1984). Enzymatic activities were expressed as one µmol of substrate oxidized per minute per gram of dry mass ($U.g^{-1}$ DM).

Differences of phenoloxidase and peroxidase pools between *Sphagnum* segments, sampling areas, seasons and climate treatments were analysed using repeated measures ANOVA with time as a within subject repeated factor (time = 2: Summer and Autumn) and warming treatment, area or sampling depth as between-subject factors. Interactions between sampling area, seasons and treatments were also considered. The assumptions of parametric tests were visualized and tested. An identical procedure was used to detect differences of air and soil temperatures between ambient and warming plots.

The highest values of phenoloxidase activities were recorded with DAF. Syringaldazine and ABTS substrates induced a formation of precipitates after 1h of enzyme kinetic. Indeed, quinones produced from syringaldazine or ABTS were not soluble over time in aqueous medium (Floch et al., 2007). Phenoloxidase activities quantified with DAF showed significant differences along *Sphagnum* segments and between fen and bog ecological areas, whereas phenoloxidases quantified with L-DOPA did not change (Fig. 1). Therefore, the use of DAF is ideal to quantify phenoloxidases in *Sphagnum* peatlands.

We showed that peroxidases constituted the main oxidative system in *Sphagnum*-peatlands, with values of peroxidase activities 1000-fold higher than those of phenoloxidases (Figs 1 and 2). Although peroxidase activities 120-fold higher than phenoloxidase activities have been recorded in surface forest litter (Alarcón-Gutiérrez et al., 2009), peroxidases 1000-fold greater than phenoloxidases is unique, to our knowledge (Sinsabaugh, 2010). Moreover,

such ratio strongly suggested that peroxidases had a plant origin for several reasons. First, *Sphagnum* lawns largely dominate fen and bog areas. Second, DAF-$H_2O_2$ is known to be the most sensitive substrate for the detection of plant-peroxidases (Criquet et al., 2000b, 2001). Third, no fungal lignin-peroxidase activity was highlighted ($< 10^{-7}$ $U.g^{-1}$ DM), corroborating the *Sphagnum* origin of our high peroxidase activities. Furthermore, among the different fungus taxa identified in *Sphagnum* litter, few (only 24%) are identified as phenoloxidase producers (Thormann et al., 2001). Abiotic conditions (e.g. acidic conditions and waterlogging) of peatlands are largely known to limit fungal oxidation activity (Williams et al., 2000; Toberman et al., 2008, 2010).

Significant decreased of peroxidases were recorded both along *Sphagnum* segments and over the seasons (ANOVA, $P < 0.01$; Fig. 2). Such temporal variations suggest a positive relationship between peroxidase activities and polyphenol content. Jassey et al. (2011a, b) actually demonstrated that phenolic release from *Sphagnum* mosses changed over seasons, and decreased along *Sphagnum* shoots.

Warming by OTCs significantly increased the daily average air temperature (ANOVA, $P < 0.001$) in both sampling areas (an average increase of 1 °C). The increase of air temperatures also induce higher evapotranspiration in temperate zones, which result in lower *Sphagnum* moisture content during summertime (Jassey et al., 2011b). Despite this rise of air temperatures, phenoloxidase activities were not significantly influenced. Previous studies, which attempted to evaluate warming effect on phenoloxidases in peatlands, found equivocal results and concluded that the interactive effects of moisture and pH largely inhibited their oxidation activity (Toberman et al., 2010; Jassey et al., 2011b). On the contrary, the rise of air temperatures led to a significant increase of peroxidase activities in the fen area (+ 30%), especially in living top segments (ANOVA, $P = 0.017$; Fig. 2). The response of peroxidase activities to climate warming between the fen and bog areas also showed that oxidative pools change in different directions in response to climate warming, as already showed with

phenolics (Jassey et al., 2011b). Although temperature was identified as an enhancer of peroxidase activities, it still remains difficult to predict the effect of global warming on soil organic matter sequestration in peatlands because of multiple functions of peroxidases, both in mineralization and humification pathways (Sinsabaugh, 2010).

To conclude, our results point out that (i) the DAF is a relevant oxidative substrate to quantify both phenoloxidase and peroxidase activities in enzymatic extract from *Sphagnum* lawns, (ii) *Sphagnum*-peroxidase activities constituted the main oxidative system in *Sphagnum*-peatlands and (iii) the monitoring of plant-peroxidases represents a suitable and forward indicator of changes in carbon cycle in peatlands under a climate warming.

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225 Figures:

226 Figure 1: Fungal phenoloxidase activities (mean ± S.E.; n = 3) characterized by DAF or L-
227 DOPA substrates along the fen-bog gradient of the Forbonnet peatland in summer and autumn
228 2010 in different *Sphagnum* segments. *Asterisks* indicate significant differences of
229 phenoloxidase activities (ANOVA; $P < 0.05$) between *Sphagnum* segments. *Letters* indicate
230 significant differences of phenoloxidase activities (ANOVA; $P < 0.05$) between the fen and
231 the bog area. Top = 0-3 cm; Bottom = 3-10 cm from capitulum.

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233 Figure 2: Activity of peroxidases produced by *Sphagnum* (mean ± S.E.; n = 3) along the fen-
234 bog gradient of the Forbonnet peatland in summer and autumn 2010 in different *Sphagnum*
235 segments. *Asterisks* indicate significant differences of peroxidase activities (ANOVA; $P <$
236 0.05) between *Sphagnum* segments. *Letters* indicate significant differences of peroxidase
237 activities (ANOVA; $P < 0.05$) between seasons (summer/autumn). Triangles (Δ) indicate
238 significant differences between ambient and warming plots. Perox = peroxidases; Mn-perox =
239 manganese peroxidases. Top = 0-3 cm; Bottom = 3-10 cm from capitulum.

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